Development of a Magnetic Immunobead Assay for the Efficient Detection of Histamine in Fish Products

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Abstract

High levels of histamine concentration, 4-(2-aminoethyl)imidazol, due to time/temperature abuse in fish and meat products have been identified as a primary source of food-related intoxication. Histamine, a heterocyclic primary amine, is the result of bacteria that produce the enzyme histidine decarboxylase, which alters the molecular structure of the amino acid L-histidine. Under the auspices of Dr. Joanne Ebesu from Oceanit, the proposal for a more efficient method of histamine detection focused primarily on refining the purification steps of the standard, three-phase approach of extraction, purification and detection. Compared to techniques currently in use, the proposed method would not only expedite and possibly enhance a lengthy enzyme linked immunosorbent assay procedure (ELISA), but it would also require less laboratory equipment involved in high-performance liquid chromatography (HPLC) separation. However, the unforeseen introduction of biogenic amines into the histamine isolation procedure compromised the purification of the toxin for accurate quantification. Current research uses a type of ELISA procedure that similarly focuses on enhancing histamine purification, and then uses a competition reaction during the detection phase. So far experiments have been initially somewhat promising, but will require significant refining.

Introduction:

High levels of histamine concentration, 4-(2-aminoethyl)imidazol, due to time/temperature abuse in fish and meat products have been identified as a primary source of food-related intoxication. Histamine, a heterocyclic primary amine, is the result of bacteria that produce the enzyme histidine decarboxylase, which alters the molecular structure of the amino acid L-histidine (Serrar *et al.*, 1994). Histamine and its association with fish decomposition, has also been implicated in scombroid fish poisoning, occasionally being mistaken for "fish allergy" (Walters, 1984). Scombroid poisoning is common in tuna, mackerel, skipjack, and bonito, members of the families Scombridae and Scomberescocidae, as well as nonscombroid fish such as mahi-mahi, bluefish, amberjack, herring, sardines, and anchovies. The symptoms of histamine poisoning can last up to 12 hours and include severe throbbing headaches, palpitations of the heart, abdominal cramps, diarrhea, flushing of the face and other parts of the body

similar to sunburn, as well as itching around the mouth, nausea and weakness. Antihistimines have been the common approach to treating these symptoms. Scombroid poisoning has not been implicated in deaths in Hawaii, and few fatalities have been reported worldwide (Epidemiology Branch, 1991).

Due to rising fish consumption in the United States, quality control through histamine detection is becoming increasingly urgent. In addition to FDA regulations regarding the handling and refrigeration of fish upon death, the nature of the histamine-producing bacteria requires further evidence of quality and freshness. Deep-sea fish allowed to struggle on fishing lines for extended periods of time increase body temperatures to levels more optimal for the bacterial growth. Furthermore, the presence of the histidine decarboxylase enzyme activates histamine production despite the bacteria having been sterilized or rendered inactive through refrigeration. Recent studies have also indicated that excessive amounts of enzyme can continue to produce histamine even after freezing. In larger fish, the histamine levels are unlikely to be uniform within the individual fish itself (U.S. Food & Drug Administration, 1998). Although some scombrotoxic fish may exceed histamine levels above 1000 ppm, the concentration is usually in excess of around 200 ppm. FDA regulations set the defect action level, where regulatory action must occur, at 50 ppm (Rogers & Staruszkiewicz, 2000).

Histamine detection techniques have most often employed fluorometric or chromatographic methods, but these have typically required expensive laboratory equipment and excessive amounts of time. Often the tests are a trade-off between relatively quick, semi-qualitative, and precise, quantitative results. Thin-layered Chromatography (TLC) is one such chromatographic test that is inexpensive and simple,

but yields only semi-quantitative results. High Performance Liquid Chromatography (HPLC), in contrast, is highly specific and can be automated, but is very protracted and requires expensive laboratory equipment (Serrar *et al.*, 1994). The most common fluorometric method derives a compound of O-phthalaldehyde (OPT) and histamine through a condensation reaction stabilized by an acidification step at low temperatures. When first utilized for histamine detection, the OPT method was problematically non-specific to histamine and would often bind to other biogenic compounds (e.g. spermidine). Conceptually, this problem was approached in two ways. The first attempted to improve the isolation and purification of histamine before OPT binding, while the second ventured to improve the specificity of the histamine assay (Hakanson *et al.*, 1971).

By the mid-90s, an enzyme-linked immunoassay (ELISA) using monoclonal antibodies was developed and significantly enhanced the specificity of the OPT fluorometric method. Since histamine is a small molecule, the development of antibodies for this technique was challenging and required a proteic carrier as well as a spacer before binding with an antibody could be achieved (Serrar *et al.*, 1994). Currently, with few exceptions, histamine detection methods have chosen to utilize ELISA to enhance the specificity of fluorometric techniques.

Due to Oceanit's experience in developing kits for rapid and simple ciguatera toxin detection, a number of possibilities were considered to duplicate and improve the efficiency of histamine detection methods presently on the market. Under the auspices of Dr. Joanne Ebesu, this MarBEC 2002 spring internship concerned itself with one of these endeavors.

It was proposed that a magnetic immunobead assay be developed in which magnetic particles would be coated with histamine antibodies and mixed in a test tube with a fish extract of an unknown amount of histamine. After allowing time for binding, an external magnet would be applied to the solution while rinse steps would allow other particles, such as biogenic compounds, to be discarded. The remaining histamine solution would then be mixed with a chemical dye that would react with histamine to produce a color change that can be quantified using a spectrophotometer or fluorometer, or semi-quantified visually using UV light.

There are a number of reasons this process may increase the efficiency of identification. To analyze a food sample using any one of the various ELISA methods currently being marketed, one must first extract the histamine molecules, isolate and purify the extraction, and finally prepare it to be assayed. This preparation period can range from 20 minutes to several hours. Once prepared, ELISA techniques can still require 6 to 10 additional steps, before histamine presence can be confirmed.

The proposed method focuses specifically on enhancing the isolation and purification of histamine before it is assayed and its concentration quantified. This approach resolves the problem of assay specificity that the ELISA method addresses and therefore could possibly reduce the number of steps in overall histamine detection by offering a more simple isolation phase.

Methods and Materials:

The method being proposed would consolidate the entire detection procedure into 6 general steps:

1. Homogenizing fish tissue. The sample would also need to be acidified and subsequently alkalized as well as undergo filtration (acid extracts base).

2. Adding buffer and magnetic-antibody, mixing and incubating.
3. Applying a magnetic field.
4. Rinsing several times.
5. Adding OPT fluorescence marker.
6. Quantifying results visually (if possible) or using a spectrophotometer.

The project was divided into the three phases of extraction (step 1), purification (steps 2-4), and detection (steps 5-6). While the innovation of the proposed technique would enhance purification, our experiments initially focused on the detection phase so that a working quantification method to evaluate the success of the histamine isolation would be established. Essentially, the test would be developed in reverse.

The histamine marker used in this phase was a standard (OPT) reaction that could be derived from a variety of published procedures. The initial attempt at an OPT reaction used a 1% solution of the aldehyde in MeOH. The published procedure (based on Vidal-Carou, *et al.* 1990) basified a sample of histamine in HCl, immediately added the OPT/methanol solution, and then stabilized the reaction with citric acid. Dr. Ebesu had previously run this experiment and obtained negative results. Based on a 1998 Frattini & Lionetti paper, the procedure was slightly modified to add a small amount of B-mercaptoethanol (BME) just before the OPT solution would be introduced. The experiment initially returned positive results. However, reproducing the fluorescent reaction on subsequent attempts was unsuccessful. The entire Frattini & Lionetti 1998 procedure was at that point considered.

The procedure used a derivatizing OPT solution with minimal amounts of methanol, BME, and borax, and an excessive amount of NaOH. It was in some ways less

complicated than the Vidal-Carou 1990 method. The only deficiency was that the OPT derivatizing solution lasted 24 hours before a new sample had to be made. Repeated experiments determined that this detection method was reliable. The results of a histamine concentration curve using this procedure can be found in the *results* section.

Having identified a reliable detection procedure, the purification phase could then be developed. The first step was to conjugate the polyclonal histamine antibody purchased from Sigma-Aldrich, to a sample of Dynabeads M-280 Tosylactivated superparamagnetic, polystyrene beads coated with a polyurethane layer. In addition to multiple washings, the conjugation required prolonged incubation times, and the process, therefore, took 3 days to complete. Ten samples, conjugated 2 months prior, were initially available.

Histamine, with its basic R group from its histidine origin, is, just as any other basic molecule, very soluble in acid. In fact, an extraction procedure of histamine from a fish sample could simply be soaking the fish in a 1N HCl solution, a pH of around 1 or 2. The histamine sample used to test the magnetic assay method had previously been extracted and purified and was still in its highly acidic HCl solution. Before the magnetic beads with conjugated antibody could be added, the acidity of the histamine solution had to be neutralized to a pH within the range of 4 to 9, a standard environment for antibody/antigen reactions. This pH was achieved using pure samples of the same buffer that was used to suspend the magnetic immunobeads. Once neutralized, the beads coated with antibody were added to the histamine solution and allowed to react for a period of 5 minutes before an external magnetic field was applied and subsequent washings

performed to isolate the histamine. Using NaOH, the sample was then alkalized to be assayed with the OPT derivatizing solution.

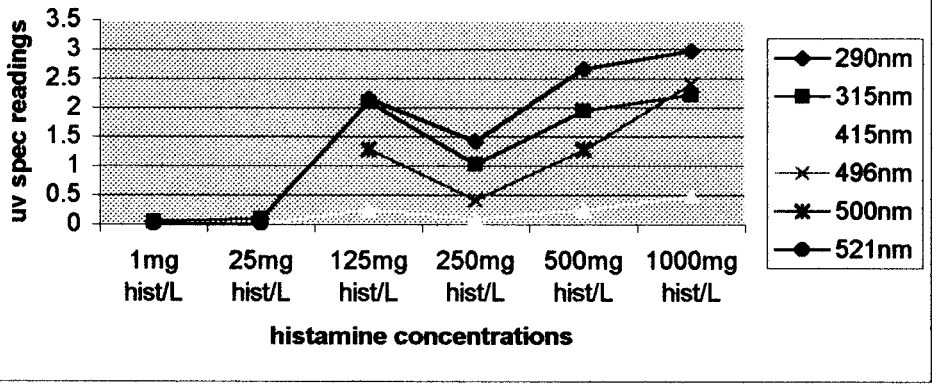
The purification procedure combined with the detection method was clearly visible under UV light. The intensity was less than those samples where histamine was exclusively assayed, but the success of the procedure was apparent. Because neither a spectrophotometer or fluorometer was available, quantification did not occur.

A new sample of immunobeads was conjugated the following week and a spectrophotometer secured. However, experimental error ruined the samples before they could be quantified. The conjugation was repeated and the samples successfully quantified using a spectrophotometer.

Results:

Each of the histamine concentrations were scanned and measured for OPT reaction at various wavelengths (Figure 1). OPT fluoresces when hit with waves in the UV range, and so the waves at 315 and 415 nanometers were the focus. The curve for these values showed that an increase in histamine concentration corresponded to an increase in measured intensity in the UV range. The 250 mg histamine/liter of HCl

Figure 1. Histamine concentration curve. Detection w/OPT derivatizing solution (based on procedure in Frattini *et al*., 1998)



concentration was an older sample and was not diluted from the original stock that the other histamine concentrations were derived from and probably deviated from the curve for that reason. The overall success of the experiment established this procedure as the method to quantify the effectiveness of the proposed isolation method. The OPT fluorescence could be visually detected with the application of UV light (Figure 2).

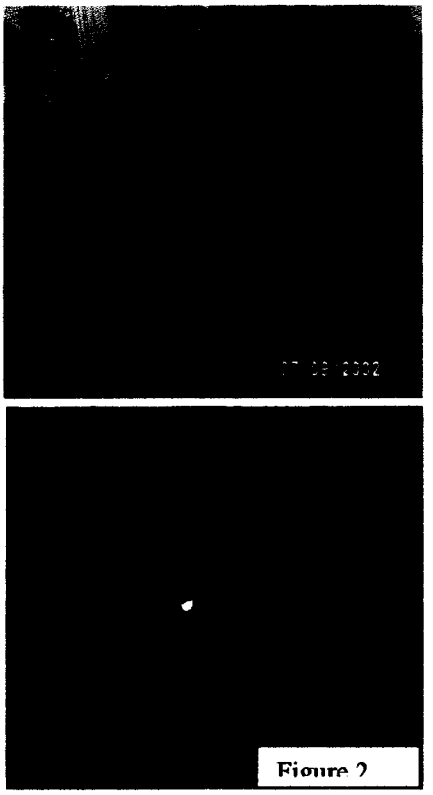


Figure 2

A spectrophotometer was also used to determine the success of integrating the conjugated immunobeads in the purification phase. The samples from a histamine concentration curve were scanned individually to see where UV sensitivity peaked (Appendix 1). As can be seen from the graphs, there was an extreme amount of "noise" recorded in each of the readings. Also, there were no clear peaks of any kind within the UV range, and the measurements for each of the concentration samples never exceeded a value of 0.003, which reading is considered extremely low and faint. The spectrophotometer readings basically did not provide evidence of increasing OPT fluorescence as histamine concentrations increased. Despite a faint positive result that could be seen visually when a UV light was applied, the samples were never measured using a fluorometer, which would have increased the sensitivity of the readings.

Discussion:

The histamine concentration curve of the immunobead purification showed that OPT fluorescence did not respond to increased histamine concentrations. There are a

number of potential reasons for these inconclusive results. One very likely possibility is that the suspended magnetic beads would have dispersed the spectrophotometer's UV light entering the sample from one end and prevented it from being recorded on the other as it passed through. A solution that was never explored involved adding a chemical that would separate the OPT-histamine-antibody molecule from the magnetic beads that assisted in the histamine purification. The company Dynal that produces the magnetic beads has developed reagents that could be compatible with a histamine-antibody conjugate.

It could also be that spectrophotometer measurements are not sensitive enough to detect the fluorescence of OPT with the interference of magnetic particles. Using a fluorometer is a conceptually simple modification. Fluorometers are significantly more sensitive than spectrophotometers, but are also much more expensive and in short supply.

The most likely reason however, that there was little UV distinction between samples with histamine and the controls without it, was that the purification step actually undid what it tried to accomplish. The polyclonal antibodies that were introduced via the magnetic beads probably had up to 15 additional amine binding sites per molecule to which OPT would have readily attached. Because there is no simple way to detach the polyclonal histamine antibodies from histamine antigen molecules as a final preparation step before OPT addition, it was determined that this method was too inefficient to expend further time or resources.

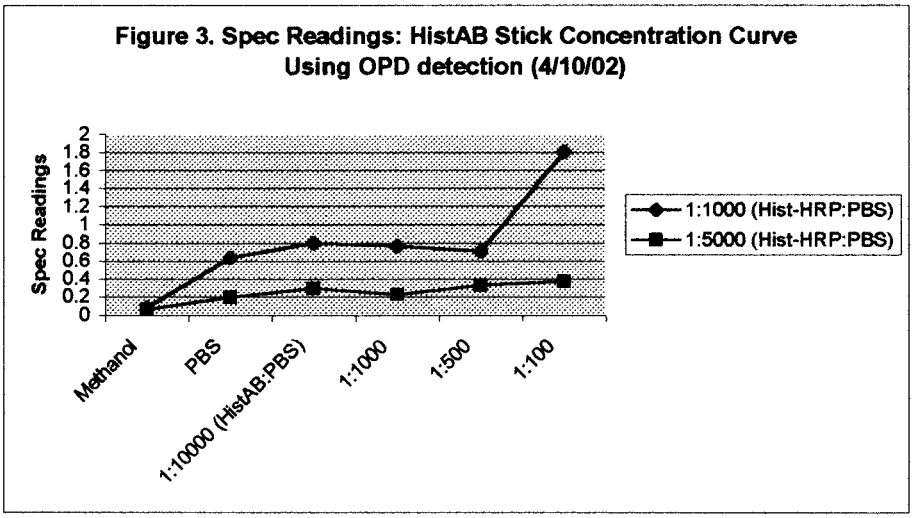
Current Research:

New research similarly focuses on modifying the isolation phase using a variation of the ELISA method. Traditional ELISA techniques attach a detection enzyme to an antibody that is then added to a solution of antigen for quantification. Current experiments at Oceanit attempt to bind the histamine antigen to the horseradish peroxidase (HRP) protein before isolating the conjugation using the polyclonal histamine antibody (Ab). If successful, this can eventually lead to a method where a known concentration of laboratory produced histamine conjugate is added to an unknown fish extracted histamine concentration. Using the isolation procedure, a reaction where the tagged laboratory histamine competes with the fish histamine for Ab binding would produce a negative curve of color intensity and would therefore indicate higher levels of histamine within the fish sample by a drop in color intensity. So far the experiments have been initially somewhat promising, but will require significant refining.

The histamine-HRP conjugate was obtained through dialysis using a membrane that allows transit for molecules with a molecular weight up to 12,000 to 14,000. The membrane with the conjugating molecules were left in a 0.1 M phosphate buffer solution (PBS) of 7.4 pH for a period around 3 days. After dialysis the conjugate was centrifuged and filtered through a sterile membrane to remove unbound molecules and was continually kept at 4 degree C to stabilize the bond. For quantification the conjugate was added to a blocking buffer of .01 M PBS at 7.4 pH mixed with bovine serum albumin (BSA) to make varying concentrations (usually 1:1000). Membranous sticks with attached histamine Ab dilutions in 7.4 pH, .01 M PBS (1:50, 1:100, 1:1000, 1:10000) were added to the conjugate-wash solution. These sticks were prepared by pre-wetting

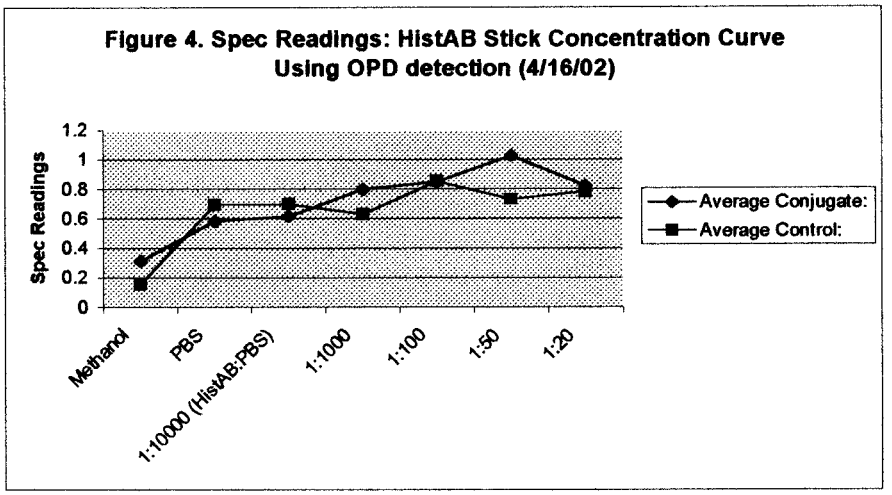
the membrane with 100% methanol before adding the antibody solution. After 10 minutes these sticks were removed and washed in a 7.4 pH, .01 M PBS solution with .05% tween20. The sticks were then placed for 10 minutes in microplate wells filled with O-phenylenediamine (OPD), a solution that produces a color change when added to HRP. After the reaction, the sticks were removed from the OPD solution and equal amounts of sulfuric acid were added to the OPD wells to discontinue the reaction. The OPD samples were then placed in a microplate spectrophotometer and measured at 450 nm. Because the OPD color reaction was caused by HRP presence in the OPD solution and was not the result of a histamine-antibody-HRP-OPD complex, an increase in solution color intensity would indicate higher concentrations of the histamine conjugate.

Initial experiments did not produce results in which the OPD color intensity increased significantly with higher histamine concentrations, and were therefore inconclusive (Figure 3). There was one high reading at 1.8 of the 1:100 Ab stick in a 1:1000 histamine solution, but all the other Ab sticks were generally low. It was speculated that the pores of the membrane at around 12,000 molecular weight were too large to contain the histamine molecules (around 150 mw) long enough to bind with HRP. It was possible



therefore, that the color reactions could simply be the reaction of HRP molecules with no attached histamine.

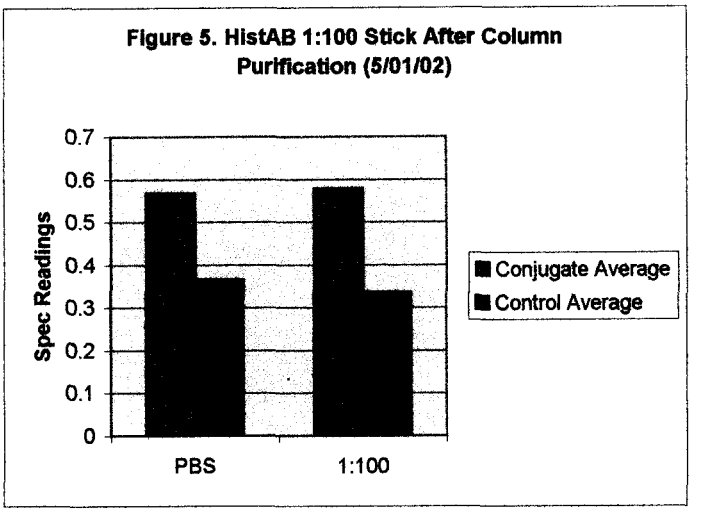
The procedure was modified to include a 3-hour incubation period with a small sample of 1% gluteraldehyde added to promote initial conjugate binding before dialysis. An HRP control containing no histamine was also dialyzed using the same procedure to verify that the Ab sticks were functioning to isolate the histamine complex. PBS and methanol controls on membranous sticks were included at 1:1000 dilutions of both histamine conjugates and HRP samples. The experiment was run in duplicate and the spectrophotometer readings averaged. The results turned out to be somewhat promising. As the membrane-bound Ab conjugate concentration rose there was an increase in color intensity (Figure 4). However, the isolation of the histamine was still not clear. The HRP control, although slightly lower in most areas, read considerably close to the histamine conjugate.

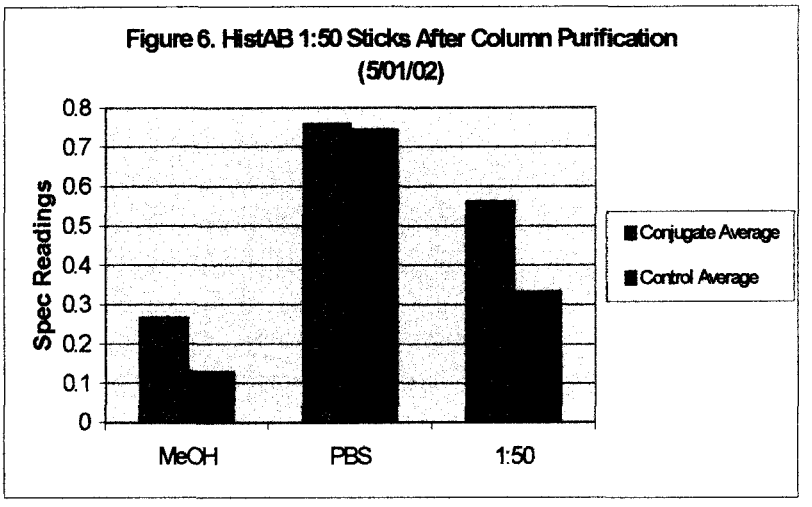


To lower the HRP control reading, both the conjugate and HRP samples went through column purification using sephadex g-10 to filter out any unbound HRP molecules. Fractions were collected and measured with a spectrophotometer to evaluate dilution. Tests were run on the most concentrated fractions of both the conjugate and control, but the results produced no trends or even subtle distinctions between varying Ab

concentrations or histamine presence. It was speculated that by this time the conjugation had degraded and that new samples had to be dialyzed. The addition of an equal amount of glycerol to the histamine-HRP complex should be included as a last step to stabilize the conjugation.

While it was clear that the conjugate at a 1:100 Ab concentration read significantly higher than its HRP control, indicating effective isolation, the PBS control membranes ran unexpectedly close to the Ab conjugates (Figure 5). The experiment was repeated using 1:50 Ab sticks and included a methanol control (Figure 6). In this experiment the PBS actually ran higher than the Ab sticks. Experiments were performed using methanol dilutions of Ab with the solution applied directly to the membrane for binding in the absence of PBS. The results showed no distinctions between the methanol-Ab membranes and the methanol controls.





Upcoming experiments will circumvent the use of membranous sticks altogether and instead us ELISA microplate wells for Ab attachment during the isolation of the

histamine conjugate. This will hopefully decrease a number of variables and potential obstacles pertaining to the use of membranes. Also, the PBS buffer used during dialysis and Ab dilution will be changed to a consistent .1 M at 7.4 pH solution. This may potentially decrease the noise of the PBS control reactions with OPD.

Evaluation of Learning:

The biotech internship experience with Oceanit this semester has been tremendously beneficial to my education in the field of biology. Participation in classroom laboratories at UH unaccompanied with this internship would have left me severely under-qualified for a career in biotech research. Many of the laboratory techniques I became familiar with are too advanced to be taught in a laboratory at the undergraduate level despite their extensive application and efficiency in addressing challenging research obstacles.

One thing in particular that impressed me was the way information for research is now gathered. Even at the most technical level, scientific articles and protocols are exchanged rapidly via the internet. Biotech companies are able to share their technology amongst themselves for quick referencing and effective research solutions.

Acknowledgements:

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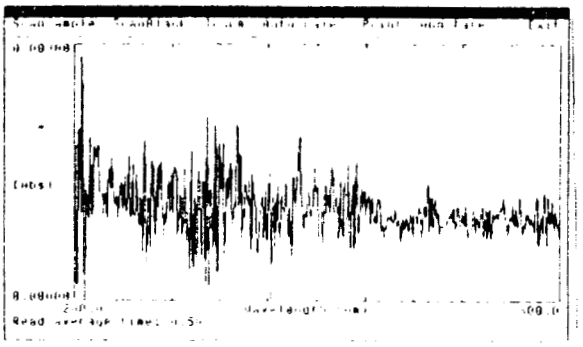
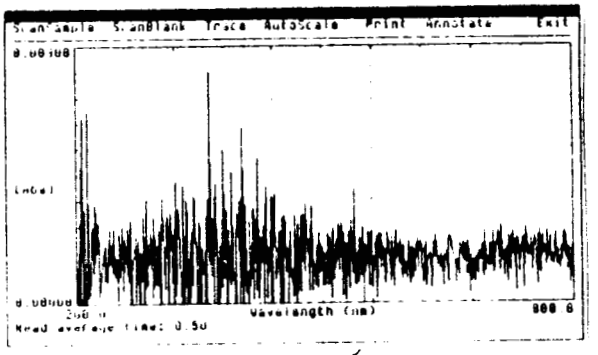
Appendices

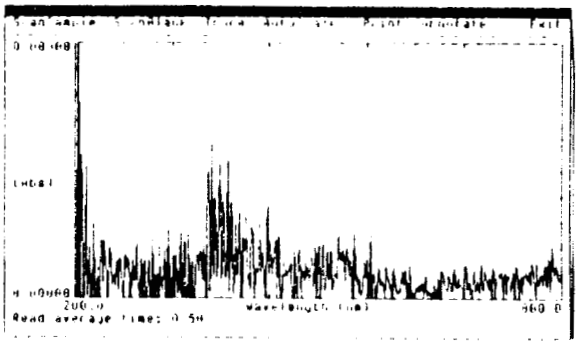
Appendix 1. Spectrophotometer scans between 200 and 800 nm of histamine concentrations isolated using a magnetic immunobead assay.



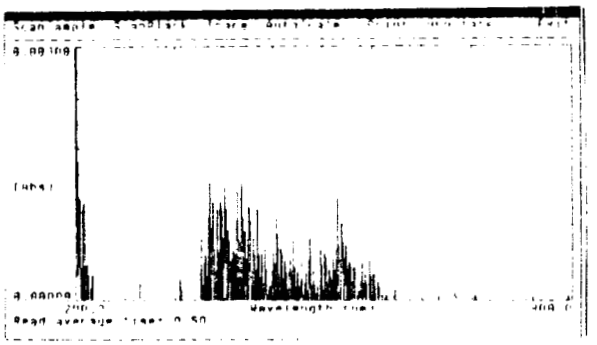
1000 mg Hist/L HCl



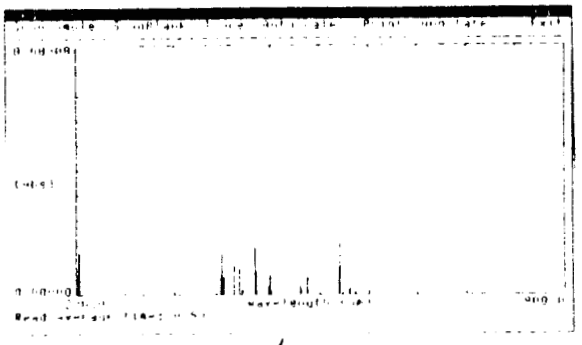
500 mg/L



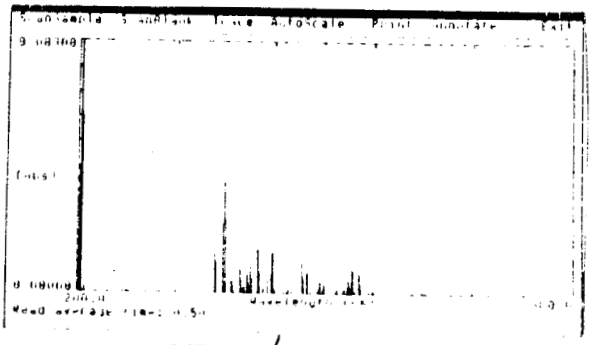
250 mg/L



100 mg/L



50 mg/L



25 mg/L